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Immunocytochemical Detection of Prolactin and Growth Hormone Cells in the Pituitary during Early Development of the Japanese Eel, *Anguilla japonica*

Emi Arakawa, Toyoji Kaneko, Katsumi Tsukamoto and Tetsuya Hirano

Ocean Research Institute, University of Tokyo
Nakano, Tokyo 164, Japan

ABSTRACT—The occurrence and development of prolactin (PRL) and growth hormone (GH) cells were investigated in the Japanese eel by means of an immunocytochemical method. Both PRL and GH cells were detected in the pituitary in all the specimens of the leptocephali, even in the smallest (10.0 mm in total length). In the leptocephalus, the mean percentage of PRL-cell area to the whole pituitary area (% PRL) was $6.5 \pm 1.2\%$, the % GH being $15.5 \pm 2.0\%$. Both % PRL and % GH exhibited a tendency to decrease as leptocephali grew larger. In the glass eel, caught just before upstream migration, the % PRL was $12.6 \pm 0.5\%$, almost twice as much as that in the leptocephalus, whereas the % GH was $16.7 \pm 1.3\%$, similar to the value in the leptocephalus. The activation of PRL cells in the glass eel suggests that PRL has an osmoregulatory role in freshwater adaptation during the upstream migration. Our findings also suggest that GH is important for larval growth and, possibly, osmoregulation in seawater during their early life stages.

INTRODUCTION

Prolactin (RPL) is well known as an important hormone for freshwater adaptation in many euryhaline species [1, 2]. Activation of PRL cells or increase in plasma PRL has been observed after transfer from seawater to fresh water. On the other hand, there is a large body of evidence that growth hormone (GH) is involved in seawater adaptation especially in salmonids. Increase in plasma GH after transfer from fresh water to seawater has been reported in several salmonid species [3–9].

The eels belong to the catadromous fishes; they experience both seawater and fresh water during their life-long migration. Ecological studies have shown that they spawn eggs offshore and leaf-shaped larvae (leptocephali) drift in the current toward the coasts. After metamorphosis, juveniles (glass eels) migrate upstream and stay in the river or the lake for 5–10 years; they grow in fresh water and return to the ocean for spawning. However, early developmental stages of the Japanese eel, *Anguilla japonica*, have hardly been studied, because of very limited availability of eggs and leptocephali. Furthermore, the spawning area of the Japanese eel had been unknown until quite recently.

In June–Jyly 1991, the expedition of the Hakuho-Maru, a research vessel of the Ocean Research Institute, University of Tokyo, succeeded in sampling more than 900 pre-leptocephali and leptocephali, and determined the spawning area of the Japanese eel to be in the North Equatorial Current west of the Mariana Islands [10]. The pre-leptocephali and leptocephali captured ranged from 7.9 to 34.2 mm in total length, much smaller and thus younger than those that had ever been collected.

In order to clarify the development of osmoregulatory mechanism during the migration of the Japanese eel, we examined the occurrence and development of both PRL and GH cells by

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means of immunocytochemistry, using valuable samples of the preleptocephali and leptocephali, together with glass eels just before upsteam migration to the river as well as sexually immature, cultured eels. This is the first report on the identification of PRL and GH cells during the early developmental stages of the Japanese eel.

MATERIALS AND METHODS

Pre-leptocephali and leptocephali of the Japanese eel (10.0–30.0 mm in total length), estimated to be 12–48 days old, were collected in the area west of Mariana Islands (salinity: 34.5‰) in June–July 1991 [10]. In the present paper, the term leptocephalus designates both preleptocephalus and leptocephalus for convenience. Glass eels of the same species (55–60 mm) were caught on the coast of Taiwan (salinity: 30‰) in November 1991. Sexually immature, cultured eels, weighing about 200 g, were obtained from a commercial dealer in Tokyo.

Head portions of leptocephali and glass eels and pituitaries of cultured eels were fixed for 24 h in Bouin's solution and preserved in 70% ethanol. Later, they were embedded in paraplast, and sagittal sections were cut serially at 4 μm thickness. To identify PRL and GH cells in the pituitary, the sections were stained immunocytochemically according to the ABC method [11] using commercial reagents (Vectastain ABC Kit, Vector Laboratories). Briefly, the sections were sequentially incubated with (1) 0.6% hydrogen peroxide (H_2O_2) for 30 min to inactivate endogenous peroxidase activity, (2) 2% normal goat serum for 30 min to reduce non-specific binding, (3) the specific antisera against eel PRL [12] and eel GH [13], overnight at 4°C, (4) biotinylated anti-rabbit IgG for 30 min, (5) avidin-biotin-peroxidase complex (ABC) for 1 h, and (6) 0.02% 3,3′-diaminobenzidine tetrahydrochloride containing 0.005% H_2O_2 for 4–5 min.

To determine the optimal dilution of the antisera, preliminary experiments were conducted, where the pituitary of the cultured eel was immunocytochemically stained with serial dilutions of the antisera against PRL and GH. The best staining was obtained at a dilution of 1 : 16000 in both PRL and GH cells. In the case of PRL cells, however, a lower dilution of 1 : 8000 was also employed, since the RPL cells in the leptocephalus were stained rather faintly at the dilution of 1 : 16000. The specificity of the immunoreaction was confirmed by preabsorbing the antisera with respective antigens.

For the quantitative analysis, percentages of PRL- and GH-cell areas to the whole pituitary area (% PRL and % GH) were estimated as follows: Serial sections at intervals of 16–20 μm in leptocephali and glass eels and about 100 μm in cultured eels were immunocytochemically stained with either anti-PRL or anti-GH sera. The areas of PRL and GH cells and the whole pituitary were measured on microphotographs with a tablet digitizer. The % PRL and % GH were calculated as percentages of the total PRL- and GH-cell areas, respectively, to the total pituitary area. Significant differences were determined by Student's *t*-test or Cochran-Cox test after *F*-test comparison of variance.

RESULTS

In the leptocephalus, the pituitary appeared as a cell cluster or mass, located beneath the hypothalamus, and was barely distinguishable as a definitive organ (Fig. 1A, B). Both PRL and GH cells were detectable in all specimens of leptocephali (10.0–30.0 mm) by immunocytochemistry. Compared with the cultured eel, however, PRL cells were faintly stained, while GH cells were similarly stained. PRL cells occurred in the rostral pars distalis in the pituitary (Fig. 1A), separated from the location of GH cells in the proximal pars distalis (Fig. 1B). The mid-sagittal section contained less than 5 PRL cells and 10–20 GH cells. The PRL cells did not form follicular structures, as typically seen in the adult form of the eel pituitary. The mean value of the % PRL was $6.5 \pm 1.2\%$, while the % GH was $15.5 \pm 2.0\%$, 2.4 times higher than the % PRL (Table 1). Both % PRL and % GH exhibited a tendency to decrease as leptocephali grew larger (Fig. 2).

The pituitary of the glass eel was morphologically more comparable to that of the cultured eel than that of the leptocephalus (Fig. 1C, D). The pituit-

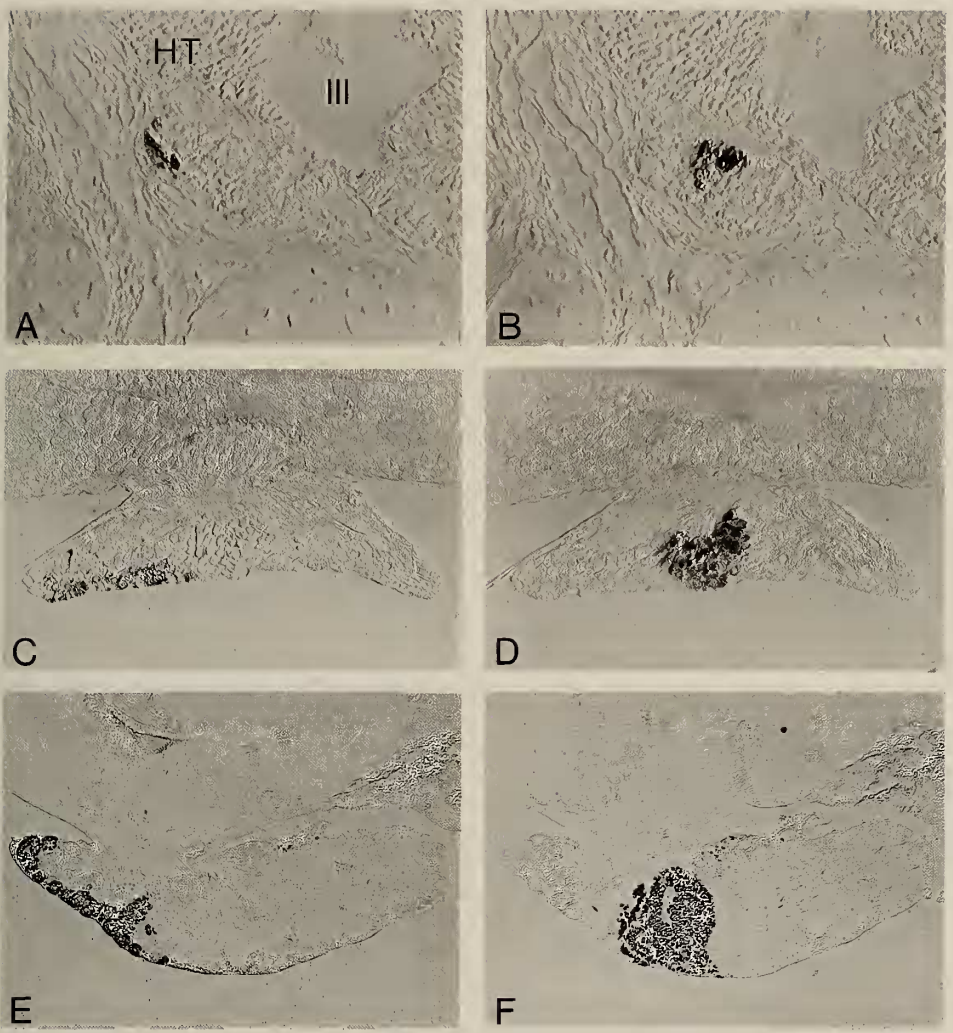

FIG. 1. Mid-sagittal sections of the pituitaries in leptocephalus (A, B; 19.7 mm in total length), glass eel (C, D) and cultured eel (E, F) of the Japanese eel, stained with anti-eel PRL (A, C, E) and anti-eel GH (B, D, F). HT, hypothalamus; III, third ventricle. Anterior to the left. A and B, ×347; C and D, ×126; E and F, ×53.

ary in this stage appeared pendent from the hypothalamus, whereas that of the leptocephalus was embedded partly in the hypothalamus. The midsagittal section contained about 30 PRL cells, gathering at the ventral edge of the rostral pars distalis, without forming follicular structures (Fig. 1C). The intensity of immunoreaction in PRL cells were still weaker than that of the cultured eel. About 40 GH cells cultured in the ventral part of the proximal part distalis (Fig. 1D). The % PRL was 12.6±0.5%, significantly higher than that in the leptocephalus, whereas the % GH (16.7± 1.3%) was similar to that in the leptocephalus (Table 1).

In cultured eels, PRL cells were located at the antero-ventral edge of the rostral pars distalis, mostly forming follicles (Fig. 1E), whereas GH cells occupied a large part of the proximal pars distalis (Fig. 1F). The % PRL in cultured eels (11.8±1.7%) was comparable to that of glass eels. In contrast, the % GH was significantly increased to 23.7±1.8%.

Table 1. Percentages of RPL-cell area (% PRL) and GH-cell area (% GH) to the whole pituitary area in different developmental stages of the Japanese eel

Percentage of cell area	Developmental stage		
	Leptocephalus	Glass eel	Cultured eel
% PRL	6.5±1.2 (6)	12.6±0.5 (6)**	11.8±1.7 (6)*
% GH	15.5±2.0 (5)	16.7±1.3 (6)	23.7±1.8 (6)*†

Values represent means±S.E. (n).
*, **, and †, significantly different from the values of leptocephali (*$P<0.05$, **$P<0.001$) and glass eels (†$P<0.05$).

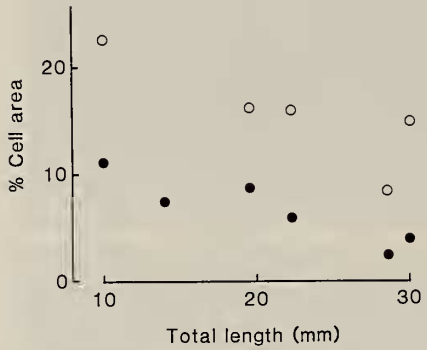

Fig. 2. Relationships between percentages of PRL- and GH-cell areas (% PRL, ●; % GH, ○) and body length in leptocephali of the Japanese eel.

DISCUSSION

In the present study, we examined for the first time the occurrence and development of PRL and GH cells in the Japanese eel, using leptocephali as well as glass eels and sexually immature, cultured eels. This study was made possible by the success of collecting a large number of leptocephali and consequent discovery of the spawaning area of the Japanese eel by the expedition of the research vessel Hakuho-Maru [10].

Both PRL and GH cells were detectable in all the leptocephali examined, even in the smallest specimen (10.0 mm) which was estimated to be 12 days after hatching. Considering the growth-promoting action of GH, it is not surprising that GH cells are present in this early developmental stage. It is of particular interest, however, that PRL cells also exist in the stage of the eel living offshore, since PRL is supporsed to be the most important hormone for freshwater adaptation in euryhaline teleosts [1, 2]. The occurrence of PRL cells in the early stages of the life has reported in a marine fish, black sea bream (*Acanthopagrus schlegeli*) [14] as well as coho salmon (*Oncorhynchus kisutch*) [15] and rainbow trout (*O. mykiss*) [16]. Our finding and others suggest that PRL is involved in some other functions than freshwater adaptation, such as larval growth; in fact it is generally accepted in amphibian that PRL is involved in larval growth [17].

Both % PRL and % GH similarly decreased as leptocephali became larger (Fig. 2), implying that PRL and GH cells develop earlier than other cell types in the pityuitary. PRL cells were more faintly stained than those in cultured eels. Although the intensity of immunocytochemical reaction does not necessarily reflect the activity of endocrine cells, PRL cells in leptocephali seem less active. This is supported by the face that the number of PRL cells, as well as the % PRL, was less in the leptocephalus in comparison with GH cells.

The % PRL increased significantly in the glass eel compared with that in the leptocephalus, although PRL cells were still faintly stained when compared with those in the cultured eel in fresh water. In cultured eels, the % PRL was similar to that in glass eels. The increase in the % PRL is well timed to upstream migration of the glass eel. Increase in PRL cells prior to migration is also observed in mullet (*Mugil cephalus*) [18, 19] and black sea bream [14]; they migrate from seawater to brackish water. In black sea bream migrating from offshore to inshore during the final phase of postflexion stage, Kimura and Tanaka [14] reported that PRL production was stimulated during the development from yolk-sac larva to juvenile, coinciding with their inshore migration. In our study, leptocephali were collected in the seawater (34.5‰), while glass eel were collected in the coastal water with a little lower salinity (30‰); the difference in environmental salinity is minor. Thus, the increase of PRL cells in the glass eel seems to be endogenously prepared in advance for freshwater adaptation or migration to the river.

The % GH was consistently higher than the %

PRL in all the leptocephali examined (Fig. 2), and the high level was maintained in glass eels. The abundance of GH cells implies their high cellular activity, and suggests that the importance of GH for larval growth and, possibly, osmoregulation in seawater. The % GH was further increased in the cultured eel, although they were in fresh water.

The pituitary was distinguishable even in the smallest leptocephalus, and became morphologically similar to the adult form of the gland in the glass eel. In the cultured eel, PRL cells typically formed follicular structures, the functional significance of which is unknown. During early developmental stages from leptocephalus to glass eel, however, PRL cells existed only as clusters of cells without forming follicles, indicating that the formation of follicular structures occurs in a later stage, presumably around the period of migration to fresh water.

In the present study, the activity of PRL cells seemed to be enhanced in the glass eel, just before upstream migration. On the other hand, GH cells remained rather active during all stages of the leptocephalus and glass eel. These findings are in consistent with the notion that PRL is responsible for freshwater adaptation, and GH for seawater adaptation as well as growth. However, further studies are required to understand the whole aspects of osmoregulatory control by PRL and GH during the early life stages of the eel.

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REFERENCES

1 Clarke, W. C. and Bern, H. A. (1980) Comparative endocrinology of prolactin, In "Hormonal Proteins and Peptides". Ed. by C. H. Li, Academic Press, New York, Vol. 8, pp. 105–197.

2 Brown, P. S. and Brown, S. C. (1989) Osmoregulatory actions of prolactin and other adenohypophysial hormones, In "Vertebrate Endocrinology: Fundamentals and Biomedical Implications". Ed. by P. K. T. Pang, M. P. Schreibman, Academic Press, New York, Vol. 2, pp. 45–84.

3 Sweeting, R. M., Wagner, G. F. and MacKeown, B. A. (1985) Changes in plasma glucose, amino acid nitrogen, and growth hormone during smoltification and seawater adaptation in coho salmon, *Oncorhynchus kisutch*. Aquaculture, **45**: 185–197.

4 Hasegawa, S., Hirano, T., Ogasawara, T., Iwata, M., Akiyama, T. and Arai, S. (1987) Osmoregulatory ability of chum salmon, *Oncorhynchus keta*, reared in fresh water for prolonged periods. Fish Physiol. Biochem., **4**: 101–110.

5 Boeuf, G., Le Bail, R. Y. and Prunet, P. (1989) Growth hormone and thyroid hormones during Atlantic salmon, *Salmo salar* L., smolting, and after transfer to seawater. Aquaculture, **82**: 257–268.

6 Collie, N. L., Bolton, J. P., Kawauchi, H. and Hirano, T. (1989) Survival of salmonids in seawater and the time-frame of growth hormone action. Fish Physiol. Biochem., **7**: 315–321.

7 Ogasawara, T., Hirano, T., Akiyama, T., Arai, S. and Tagawa, M. (1989) Changes in plasma prolactin and growth hormone concentrations during freshwater adaptation of juvenile chum salmon (*Oncorhynchus keta*) reared in seawater for a prolonged period. Fish Physiol. Biochem., **7**: 309–313.

8 Hirano, T., Ogasawara, T., Hasegawa, S., Iwata, M. and Nagahama, Y. (1990) Changes in plasma hormone levels during loss of hypoosmoregulatory capacity in mature chum salmon (*Oncorhynchus keta*) kept in seawater. Gen. Comp. Endocrinol., **78**: 254–262.

9 Sakamoto, T., Ogasawara, T. and Hirano, T. (1990) Growth hormone kinetics during adaptation to hyperosmotic environment of rainbow trout, *Salmo gairdneri*. J. Comp. Physiol. B, **160**: 1–6.

10 Tsukamoto, K. (1992) Discovery of the spawning area for Japanese eel. Nature, **356**: 789–791.

11 Hsu, S. M., Raine, L. and Fanger, H. (1981) Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabeled antibody (PAP) procedures. J. Histochem. Cytochem., **29**: 577–580.

12 Suzuki, R. and Hirano, T. (1991) Development of a homologous radioimmunoassay for eel prolactin. Gen. Comp. Endocrinol., **81**: 403–409.

13 Kishida, M. and Hirano, T. (1988) Development of radioimmunoassay for eel growth hormone. Nippon Suisan Gakkaishi, **54**: 1321–1327.

14 Kimura, R. and Tanaka, M. (1991) Prolactin production during larval and early juvenile periods of euryhaline marine fish, black sea bream, *Acanthopagrus schlegeli*. Nippon Suisan Gakkaishi, **57**: 1833–1837.

15 Leatherland, J. F. and Lin, L. (1975) Activity of the putuitary gland in embryo and larval stages of coho

salmon, *Oncorhynchus kisutch*. Can. J. Zool., **53**: 297–310.

16 Nozaki, M., Tatsumi, Y. and Ichikawa, T. (1974) Histological changes in the prolactin cells of the rainbow trout, *Salmo gairdneri irideus*, at the time of hatching. Annot. Zool. Japon., **47**: 15–21.

17 White, B. A. and Nicoll, C. S. (1982) Hormonal control of amphibian metamorphosis. In "Metamorphosis". Ed. by L. I. Gilbert, E. Frieden, Plenum Press, New York, pp. 363–396.

18 Abraham, M. (1971) The ultrastructure of the cell types and of the neurosecretory innervation in the pituitary of *Mugil cephalus* L. from fresh water, the sea, and a hypersaline lagoon. Gen. Comp. Endocrinol., **17**: 334–350.

19 Blanc-Livni, N. and Abraham, M. (1970) The influence of environmental salinity on the prolactin and gonadotropin-secreting regions in the pituitary of *Mugil* (Teleostei). Gen. Comp. Endocrinol., **14**: 184–197.